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Deletions in the S1 domain of Rrp5p cause processing at a novel site in ITS1 of yeast pre-rRNA that depends on Rex4p

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ABSTRACT

Rrp5p is the only protein so far known to be required for the processing of yeast pre-rRNA at both the early sites A0, A1 and A2 leading to 18S rRNA and at site A3, the first step specific for the pathway leading to 5.8S/25S rRNA. Previous *in vivo* mutational analysis of Rrp5p demonstrated that the first 8 of its 12 S1 RNA-binding motifs are involved in the formation of the 'short' form of 5.8S rRNA ($5.8S_S$), which is the predominant species under normal conditions. We have constructed two strains in which the genomic *RRP5* gene has been replaced by an *rrp5* deletion mutant lacking either S1 motifs 3–5 (*rrp5-Δ3*) or 5–8 (*rrp5-Δ4*). The first mutant synthesizes almost exclusively $5.8S_L$ rRNA, whereas the second one still produces a considerable amount of the $5.8S_S$ species. Nevertheless, both mutations were found to block cleavage at site A3 completely. Instead, a novel processing event occurs at a site in a conserved stem–loop structure located between sites A2 and A3, which we have named A4. A synthetic lethality screen using the *rrp5-Δ3* and *rrp-Δ4* mutations identified the *REX4* gene, which encodes a non-essential protein belonging to a class of related yeast proteins that includes several known 3′→5′ exonucleases. Inactivation of the *REX4* gene in *rrp5-Δ3* or *rrp-Δ4* cells abolished cleavage at A4, restored cleavage at A3 and returned the $5.8S_S$:$5.8S_L$ ratio to the wild-type value. The *sl* phenotype of the *rrp5Δ/rex4*$^-$ double mutants appears to be due to a severe disturbance in ribosomal subunit assembly, rather than pre-rRNA processing. The data provide direct evidence for a crucial role of the multiple S1 motifs of Rrp5p in ensuring the correct assembly and action of the processing complex responsible for cleavage at site A3. Furthermore, they clearly implicate Rex4p in both pre-rRNA processing and ribosome assembly, even though this protein is not essential for yeast.

INTRODUCTION

Ribosome production is one of the major metabolic activities in all types of cells and this process has been highly conserved during evolution. The mature rRNA species (with the exception of eukaryotic 5S rRNA) are encoded by multiple, polycistronic units that each give rise to a single precursor transcript. This transcript then undergoes a complex series of modifications including methylation and pseudouridylation as well as endo- and exonucleolytic cleavages to remove the transcribed spacer regions. Concomitantly, the various precursor intermediates are assembled with the ribosomal proteins in an ordered fashion. In eukaryotes the majority of these reactions take place in the nucleolus and require the participation of numerous non-ribosomal, *trans*-acting factors.

The processing pathway leading to the eukaryotic, mature rRNA species is best characterized in the yeast *Saccharomyces cerevisiae* (1–3; Fig. 1). The genome of *S.cerevisiae* contains 150–200 tandemly repeated rDNA units, located on chromosome XII, each consisting of an 18S, 5.8S and 25S rRNA gene separated by two internal transcribed spacers (ITS1 and ITS2) and flanked by the 5′- and 3′-external transcribed spacers (ETS; Fig. 1A). Transcription of an rDNA repeat by RNA polymerase I results in a pre-rRNA that is co-transcriptionally processed at its 3′-end by Rnt1p to produce the 35S precursor (4,5). Cleavage of the 35S pre-rRNA at the early sites A0 within the 5′-ETS, A1, the mature 5′-end of 18S rRNA, and A2 within ITS1 then gives rise to the 20S and $27SA_2$ precursor species. Components essential for these processing cleavages include several snoRNP species as well as a host of protein factors, of which the precise role of many is still unclear (1,2,6). Inactivation of any of these *trans*-acting

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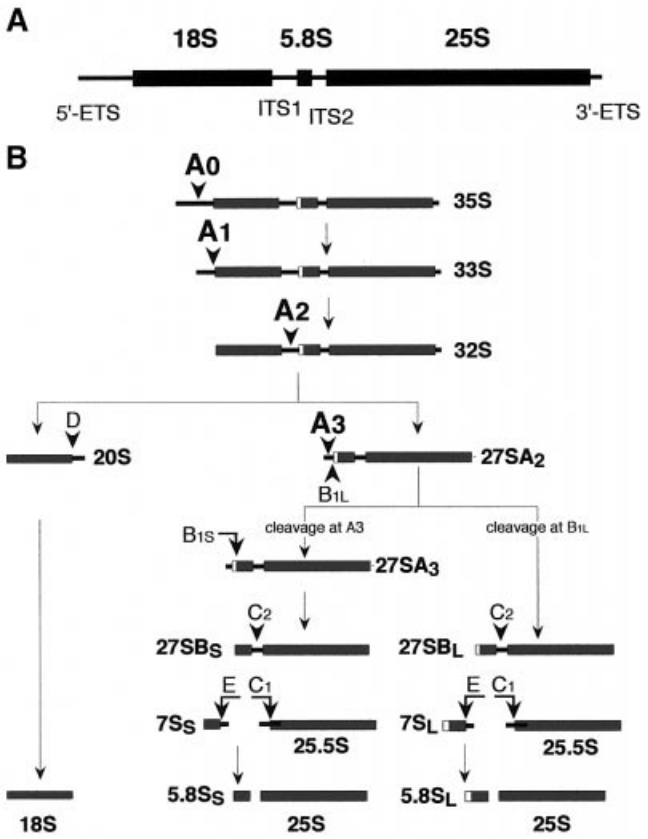


Figure 1. Processing of pre-rRNA in *S.cerevisiae*. (**A**) Structure of the rDNA transcription unit. Mature rRNA sequences are indicated by the thick bars, transcribed spacer sequences by the thin lines. (**B**) The pre-rRNA processing pathway. The processing cleavages requiring Rrp5p are show in large type. Straight arrows indicate endonucleolytic cleavages, kinked arrows represent exonucleolytic processing events. Note that the manner in which the $27SA_2$ pre-rRNA is converted into $27SB_L$ is not known.

factors inhibits formation of mature 18S rRNA indicating a strong functional interdependence.

The 20S pre-rRNA is exported to the cytoplasm where it is cleaved at site D to remove the remaining portion of ITS1 and generate the mature 18S rRNA (7,8). The majority (~90%) of the $27SA_2$ precursor undergoes endonucleolytic cleavage at site A3 by RNaseMRP (9–12). The resulting $27SA_3$ pre-rRNA is further processed by the 5′→3′ exonucleases Rat1p and Xrn1p into the $27SB_S$ species having the mature 5′-end of $5.8S_S$ rRNA (13,14). The remaining 10% of the $27SA_2$ pre-rRNA is converted into the $27SB_L$ precursor, which has the 5′-end of $5.8S_L$ rRNA located 6 nt upstream from that of its $5.8S_S$ counterpart. The precise mechanism of this alternative processing pathway is unknown. It is still unclear why eukaryotic cells contain two forms of 5.8S rRNA. However, differences in the pattern of translation have been observed between yeast cells having the normal, high $5.8S_S$:$5.8S_L$ ratio and those in which this ratio is reversed (10).

Both $27SB_S$ and $27SB_L$ pre-rRNA are further processed in an identical manner. Cleavage at site C2 within ITS2 separates the 7S and 25.5S pre-rRNAs (15). The former is converted into 5.8S rRNA by multistep 3′→5′ exonucleolytic trimming, which involves the exosome (16–18) as well as the small exonucleases Rex1p and Rex2p (19). The portion of ITS2 still present in the 25.5S precursor is removed exonucleolytically by Rat1p and Xrn1p (15). Again, a considerable number of additional *trans*-acting factors have been implicated in these steps (1,2,20–27)

Although under normal conditions RNaseMRP acts upon the $27SA_2$ precursor, it is also able to use the 35S pre-rRNA as its substrate when cleavage at the early sites is blocked (1,2). Thus, the set of *trans*-acting factors required for these early cleavages on the one hand and by RNaseMRP on the other appear to be able to act independently from each other. This conclusion is supported by the fact that correct formation of 18S and 5.8S/25S rRNA occurs even if the genes are encoded in *trans* (28). Nevertheless, there is evidence for interdependence between formation of the small and large subunit rRNAs both from mutational analysis of ITS1 (29) and experiments involving genetic depletion of the snR30 snoRNP (13). A direct link between the processing reactions required for 18S and 5.8S rRNA formation was established with the identification of the yeast *RRP5* gene (30). Genetic depletion of Rrp5p inhibits production of both 18S and $5.8S_S$ rRNA by blocking cleavage at sites A0, A1 and A2 as well as A3. The 193 kDa Rrp5p protein has a bipartite structure: the N-terminal two-thirds contain 12 S1 RNA-binding motifs, while the remaining C-terminal region consists mainly of seven tetratricopeptide (TPR) motifs (31,32). *In vivo* mutational analysis has established a direct correlation between this domain structure and the function of Rrp5p in formation of the 18S and $5.8S_S$ rRNAs, respectively. Mutations in some of the TPR motifs block the former, whereas deletions in the S1 domain strongly reduce the latter (31,32), but stimulate the production of $5.8S_L$ rRNA, which then becomes the dominant species in the mutant cells. We have now further analyzed the effect on pre-rRNA processing of two of these mutations, *rrp5-Δ3*, lacking motifs 3–5, and *rrp5-Δ4*, lacking motifs 5–8, which change the $5.8S_S$:$5.8S_L$ ratio to different extents.

MATERIALS AND METHODS

Strains and plasmids

Strain YJV154 carrying a genomic, wild-type copy of the *RRP5* gene under control of the *GAL* promoter has been described previously (31). Strains YJV306 and YJV515 were obtained by respective replacement of the wild-type *RRP5* gene of strain YJV140 by either the *rrp5-Δ3* or *rrp-Δ4* allele constructed previously (31). Strain FVY8C (*GAL-RRP5/rex*$^-$) was obtained by crossing YJV154 with YAV41 (kindly provided by Dr van Hoof), in which the *REX4* gene had been inactivated by replacing it with the kanamycin gene (19), and selecting for spores that were prototrophic for uracil (the marker for the *GAL-rrp5* gene) and resistant against geneticin. Strain yRP840, the parent strain of YAV41 was also obtained from Dr van Hoof. Cloning and transformation procedures were essentially as described previously (31).

Isolation of *sl* mutants and identification of the gene

Isolation of *sl* mutants was performed essentially as described previously (33). Strain YJV515 carrying the *rrp5-Δ4* allele on its genome, as well as the *ade2*, *ade3* and *ura3* markers, was transformed with plasmid pHT-RRP5 containing the wild-type *RRP5*, as well as the *ADE3* and *URA3* genes.

Transformants were irradiated with 254 nm UV light at a dose resulting in ~50% survival. A total of about 900 non-sectoring (red) colonies were selected by microscopic screening of approximately 1.9×10^5 survivors. After growth on yeast extract/peptone/dextrose (YPD) plates, red colonies were restreaked on plates containing 5-fluoroorotic acid (5-FOA). Thirty-five colonies unable to grow on these plates because of their inability to lose the pHT-RRP5(*URA3*) plasmid were obtained. These strains were then transformed with a plasmid containing either the wild-type *RRP5* gene or the *rrp5-Δ4* mutant allele and 16 strains in which the former, but not the latter, gene restored sectoring were selected.

The selected strains were transformed with the pUN100 yeast genomic library. About 8×10^4 colonies were screened for restoration of sectoring resulting in 20–50 candidates for each of the *sl* mutants tested. Further selection on 5-FOA plates reduced the number to 20–30 candidates per strain. To exclude strains containing a library-encoded *RRP5* gene the *Bgl*II restriction pattern of the recovered library plasmids was compared with that of pHT-RRP5. The plasmids were analyzed by PCR using *RRP5*-specific primers. About 25% of the recovered plasmids indeed contained the wild-type *RRP5* gene.

Characterization of several library plasmids recovered from *sl* strain Y11A6 identified a 10 kb fragment originating from chromosome XV, containing five complete open reading frames (ORFs). Further subcloning and testing of the resulting plasmids for their ability to restore sectoring in Y11A6 resulted in limitation to a fragment containing the overlapping ORFs YOL079 and YOL080 present on the Watson and the Crick strand, respectively. A deletion construct removing a large portion of YOL080 without affecting YOL079 proved unable to restore sectoring, demonstrating that the former gene is the one responsible for the *sl* phenotype.

RNA analysis

Isolation of RNA, northern hybridization and primer extension analysis were carried out as described previously (34). The position of the primers is indicated in Figure 2A. The sequences of these primers are given in Table 1.

Sucrose gradient analysis

Strains YJV154 or FVY88C transformants were grown on minimal medium with galactose as the carbon source and shifted to glucose-based medium. After 24 h, cycloheximide was added at the final concentration of 50 µg/ml and the cells were cooled to 0°C. Cells were harvested by centrifugation, washed with 10 ml lysis buffer [20 mM HEPES pH 7.4, 100 mM KCl, 2 mM $MgAc_2$, 50 µg/ml cycloheximide, 200 µg/ml heparin, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF)] and stored at –20°C. Extracts were prepared by adding 1 ml of lysis buffer to the cell pellet and vortexing with 0.5 mg glass beads 10 times for 30 s with intermittent cooling on ice. The resulting lysate was cleared by centrifuging twice in an Eppendorf centrifuge at 13 000 r.p.m. for 20 min and the OD_{260} was determined. About 20 OD_{260} units of extract were layered on 15–35% sucrose gradients in 10 mM Tris–HCl pH 7.4, 70 mM NH_4Cl, 4 mM $MgAc_2$, 1 mM DTT, and the gradients were centrifuged for 17 h at 18 000 r.p.m. in an SW28 rotor (Beckman Instruments) at 4°C. For analysis of polysome profiles we used 10–50% gradients followed by centrifugation for 17 h at 13 000 r.p.m. Gradients were analyzed by continuous scanning of the OD_{254}.

Table 1. Oligonucleotides used for northern hybridization and primer extensions analysis

No.[a]	Sequence (5′→3′)
1	GCTCTCATGCTCTTGCCAAAAC
2	GATATGAAAACTCCACAGTG
3	TTTGGGCATTCGAGCAATCGG
4	CCAGTTACGAAAATTCTTGTTTTTGAC
5	CTGCGTTCTTGATCGATGCG
6	GAATGTTTGAGAAGGAAATGACGCTC
7	CGCCTAGACGCTCTCTTCTTA

[a]The positions of the oligonucleotides are shown in Figure 2A.

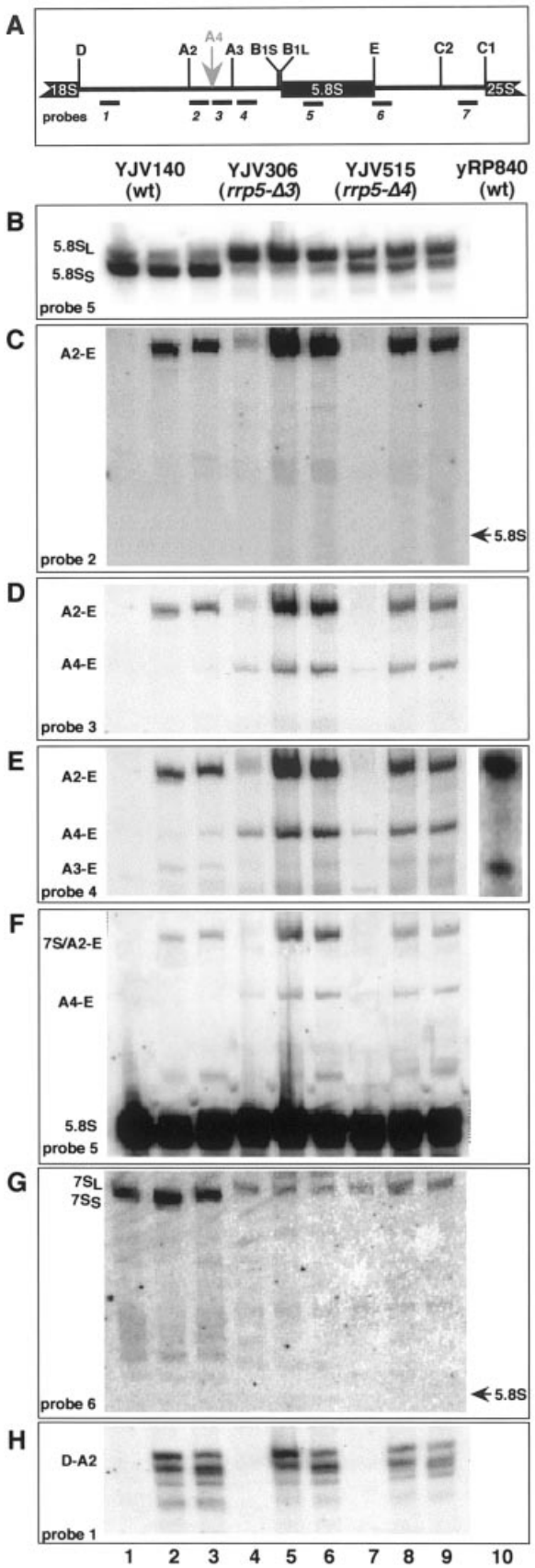
RESULTS

The *rrp5-Δ3* and *rrp5-Δ4* deletions shift processing within ITS1 from site A3 to a novel, upstream site

Using a yeast strain in which the genomic *RRP5* gene is conditionally expressed under control of the *GAL* promoter, we have shown that deletion of S1 motifs 1–2 (*rrp5-Δ2*), 3–5 (*rrp5-Δ3*) or 5–8 (*rrp5-Δ4*) from the N-terminal region of the Rrp5p protein lowers production of $5.8S_S$ rRNA to different extents. At the same time formation of its $5.8S_L$ counterpart is increased to approximately the same extent (31). The first two mutations also lowered the growth rate ~2-fold, whereas the *rrp5-Δ4* mutation did not significantly affect growth. We, therefore, decided to use the *rrp5-Δ3* and *rrp5-Δ4* mutations to study the role of Rrp5p in the formation of 5.8S rRNA further. As neither deletion is lethal we constructed mutant yeast strains in which the genomic copy of the *RRP5* gene had been replaced by either of the mutant alleles (see Materials and Methods). The resulting YJV306 (*rrp5-Δ3*) and YJV515 (*rrp5-Δ4*) strains were found to grow 2 and 1.4 times more slowly than the parent (YJV140) wild-type strain, respectively (data not shown). In both cases this is slightly below the rate observed previously for yeast cells that have been switched from wild-type to the respective mutant Rrp5p by transfer from galactose- to glucose-based medium. This difference is probably due to residual production of wild-type Rrp5p in cells containing the *GAL-rrp5* gene even when grown on glucose, whereas the integrants of course do not contain any wild-type protein.

Total RNA was isolated from exponentially growing YJV140, YJV306 and YJV515 cells and subjected to northern analysis. Because the two *rrp5-Δ* mutations predominantly affect synthesis of $5.8S_S$ rRNA (31), we focused our attention on processing within ITS1, using the set of probes depicted in Figure 2A. RNA was also isolated after treating the cultures with LiCl for 2 and 6 h to facilitate detection of 5′-extended forms of 5.8S rRNA (35).

The results of the northern analysis are depicted in Figure 2B–H. In agreement with our previous results (31), YJV306 contains only a very small amount of the $5.8S_S$ species, while the level of $5.8S_L$ is increased ~10-fold (Fig. 2B, lanes 4–6). The *rrp5-Δ4* mutant also shows a clear predominance of $5.8S_L$ rRNA, but still produces a considerable amount of the $5.8S_S$ species (Fig. 2B, lanes 7–9). Again the effects of the integrated *rrp5-Δ3* and *rrp5-Δ4* mutant alleles



are more pronounced than seen previously in the carbon-source shift experiments.

Addition of LiCl to yeast cells results in the accumulation of 5′-extended forms of 5.8S rRNA because LiCl strongly inhibits the 5′→3′ exonucleases Rat1p and Xrn1p, responsible for the $27SA_3 \rightarrow 27SB_L$ processing step, as well as, to a lesser extent, RNase MRP which converts $27SA_2$ into $27SA_3$ pre-rRNA (35). However, cleavage of the $27SA_2$ and $27SA_3$ precursor species within ITS2 and the subsequent removal of the ITS2 spacer sequences from 7S pre-rRNA can still proceed. Figure 2C shows that upon LiCl treatment both the wild-type strain and the *rrp5* deletion mutants indeed accumulate a product that hybridizes to probe *2*, located between site A2 and A3, and that according to its mobility corresponds to the A2-E fragment. The same product is also recognized by probes *3* and *4* (Fig. 2D and E). Probe *6* (Fig. 2G) should not detect the A2-E fragment because it hybridizes only to RNA molecules that still contain at least a portion of ITS2. The band seen in Figure 2G, therefore, represents 7S pre-rRNA, which has almost the same size as the A2-E fragment. Probe *5* (Fig. 2F) detects both the A2-E fragment and 7S pre-rRNA. Furthermore, we can also detect the complementary D-A2 fragment of ITS1 in all three strains upon hybridization with probe *1* (Fig. 2H). Somewhat unexpectedly, we did not observe accumulation of the A3-E fragment upon hybridization with probes *4* or *5* (Fig. 2E and F). We surmise that this is due to the genetic makeup of our strains, because, as shown in Figure 2E (lane 10), the A3-E fragment could be visualized by probe *4* in another *RRP5* wild-type strain, yRP840 (19), after treatment with LiCl for 2 h. Interestingly, however, probes *4* and *5* did detect a fragment in both the *rrp5-Δ3* and *rrp5-Δ4* strains that is absent from the wild-type cells (Fig. 2E and F, lanes 4–9). The same fragment also hybridizes with probe *3*, complementary to the region of ITS1 directly upstream from site A3 (Fig. 2D, lanes 4–9), but not with either probe *2* (Fig. 2C) or probe *6* (Fig. 2G). The data indicate that in the *rrp5-Δ3* and *rrp5-Δ4* cells a novel processing event occurs within ITS1, either in addition to or instead of cleavage at A3. As judged from the electrophoretic mobility of the novel fragment its 5′-end, which we have called A4, is located approximately midway between A2 and A3.

In order to establish whether processing at A4 indeed replaces that at A3 and to map the location of the A4 site more

Figure 2. Northern analysis of low-molecular-weight processing intermediates in *rrp5-Δ3* and *rrp-Δ4* cells. Cultures of strains YJV306 (*rrp5-Δ3*), YJV515(*rrp5-Δ4*) and the wild-type parental strain YJV140 were grown on YPD medium to mid-exponential phase. Samples containing identical amounts of cells were taken before addition of 0.2 M LiCl and after further incubation in the presence of LiCl for 2 and 6 h. Total RNA was isolated, separated on 8% polyacrylamide gels and blotted onto nitrocellulose filters which were then hybridized in sequence with the probes indicated in (A). (**A**) The region of the pre-rRNA spanning ITS1, 5.8S rRNA and part of ITS2, including the processing sites. The position of the different oligonucleotides used for northern hybridization and primer extension experiments is indicated. The sequences of these oligonucleotides are shown in Table 1. (**B**–**H**) Northern hybridization. Lanes 1–3, YJV140; lanes 4–6, YJV306; lanes 7–9, YJV515. Lanes 1, 4 and 7, samples taken before addition of LiCl; lanes 2, 5 and 8, samples taken 2 h after addition of LiCl; lanes 3, 6 and 9, samples taken 4 h after addition of LiCl. Lane 10 in (E) shows hybridization of probe *4* to RNA isolated from the wild-type strain yRP840 (19) 2 h after addition of LiCl.

precisely, we carried out primer extension experiments on total RNA isolated from wild-type, *rrp5-Δ3* and *rrp5-Δ4* cells either before or after treatment with LiCl using probe *4*. As shown in Figure 3A a stop corresponding to A3 is readily detectable in the wild-type strain upon LiCl treatment. In the two mutant strains, however, this stop has disappeared completely, indicating that cleavage at A3 is blocked completely. Instead, a new set of stops appears 34–36 nt farther upstream. Together with the results of the northern hybridization shown in Figure 2 the data firmly establish the occurrence of an alternative-processing event within ITS1 in the *rrp5* deletion mutants. Furthermore, our results show that there is no absolute link between cleavage at A3 and the formation of $5.8S_S$ rRNA. Despite the absence of any detectable processing at site A3 in the *rrp5-Δ4* mutant, in these cells ~40% of 5.8S rRNA still is the short form, which, therefore, should originate from the $27SA_2$ and/or $27SA_4$ intermediate. The existence of such a $27SA_4$ processing intermediate was corroborated by primer extension experiments using probes *6* and *7*, located upstream and downstream of site C2 within ITS2, respectively (Fig. 2A). In both cases we observe a clear signal corresponding to site A4 in the samples isolated from LiCl-treated *rrp5-Δ3* and *rrp5-Δ4* mutant cells, while the signal corresponding to site A3 has disappeared. In particular the detection of site A4 with probe *7* is convincing evidence for the presence of a $27SA_4$ precursor.

As shown in Figure 4 the A4 processing site maps to a region of the spacer that has been well conserved with respect to both primary and secondary structure in a wide variety of yeast species. The A4 site is located within a 5′-UCUUUG-3′ consensus sequence that is part of a stem shared by all known yeast ITS1 regions. There is no obvious structural similarity between the A4 site and either the A2 or the A3 site.

Synthetic lethality with *rrp5-Δ4* identifies the *REX4* gene

In order to obtain more information on the manner in which Rrp5p functions in pre-rRNA processing we carried out a synthetic lethality screen with the *rrp5-Δ4* mutation using the *ade2/ade3* red-white colony sectoring assay, essentially as described previously (33) (see Materials and Methods for details). Strain YJV515 carrying the *rrp5-Δ4* mutation as well as the wild-type *RRP5* gene on an *ADE3/URA3* plasmid were mutagenized by UV irradiation and surviving colonies were screened microscopically for non-sectoring mutants, indicating the inability to lose the plasmid. The presence of a mutation that is *sl* with the *rrp5-Δ4* deletion was then confirmed by streaking the non-sectoring mutants onto plates containing 5-fluorouracil which should inhibit growth because of the obligatory presence of the *URA3* plasmid carrying the wild-type *RRP5* gene. In this manner we isolated 16 mutants that regained sectoring when transformed with a wild-type *RRP5* gene but not with an empty vector, demonstrating that they carry an unlinked mutation, which is *sl* with the *rrp5-Δ4* mutation. These mutations were then characterized by transforming the strains in question with a yeast genomic library and recovering the library plasmid from resectoring colonies. Library plasmids containing the wild-type *RRP5* gene were identified by PCR analysis using *RRP5*-specific primers. Restriction mapping and sequence analysis of the remaining plasmids recovered from one of the *sl* strains (Y11A6) identified a 10 kb region of chromosome XV containing two

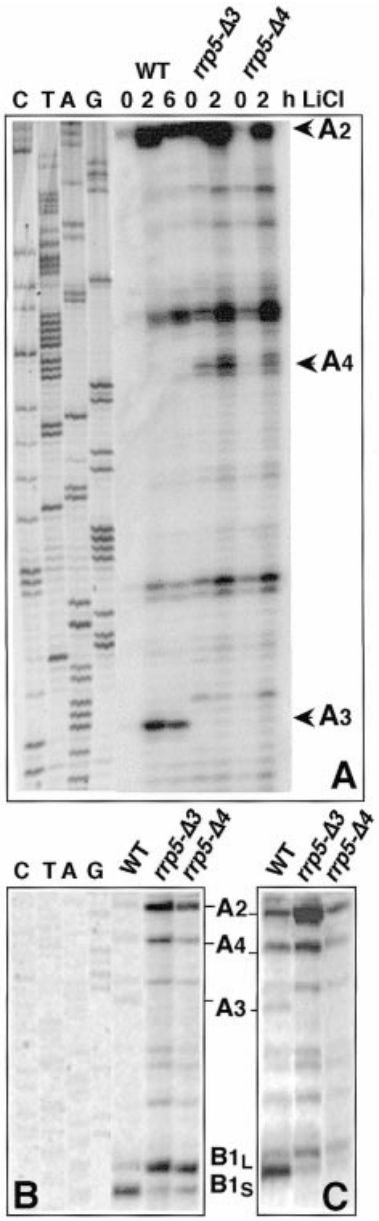


Figure 3. Primer extension analysis of precursor intermediates in the *rrp5-Δ3* and *rrp-Δ4* cells. Total RNA was isolated from exponentially growing wild-type and mutant cells immediately before and 2 h after addition of 0.2 M LiCl. Primer extension analysis was carried out using probes *4* (**A**), *6* (**B**) or *7* (**C**; see Fig. 2A for location of the probes). The positions of the primer extension stops corresponding to processing sites A2, A3 and A4 are indicated. In (B) and (C) only the samples from LiCl-treated cells are shown.

partial (*IRA2* and *DEC1*) and four complete ORFs (YOL077, YOL078, YOL079, *ATP19* and YOL080). By subcloning portions of this region the ability to restore sectoring in the Y11A6 strain was limited to a fragment that contained only ORFs YOL079 and YOL080, which are present on opposite DNA strands and overlap almost completely. To distinguish between these two ORFs, we first created a deletion that removed a large C-terminal portion of YOL080 without affecting YOL079. The resulting plasmid was unable to restore sectoring in Y11A6 cells. Secondly, mutation of the translation start codon of YOL079 did not affect the ability of

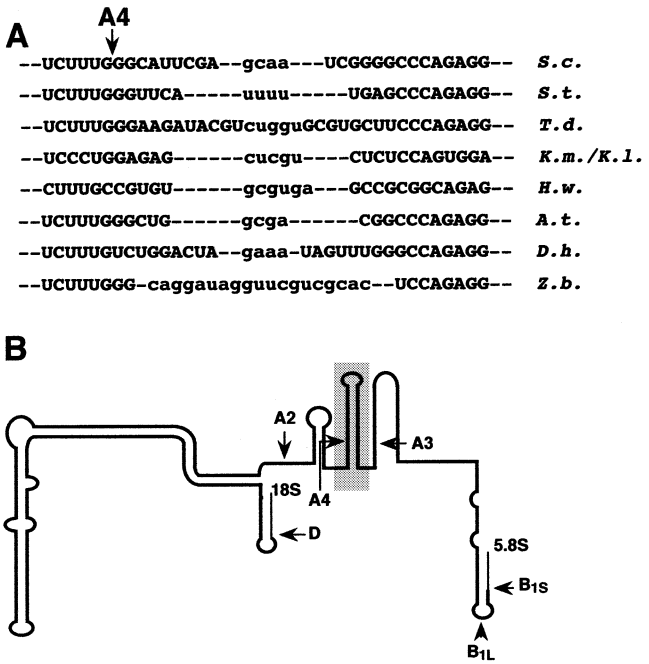


Figure 4. Structural conservation around site A4 among various yeast species. (**A**) Sequence comparison of the region of ITS1 spanning site A4 that is highlighted in (B). Nucleotides in upper case are part of the stem, nucleotides in lower case form the loop of the conserved hairpin structure containing site A4. *S.c.*, *S.cerevisiae*; *S.t.*, *Saccharomyces transvaalensis*; *T.d.*, *Torulospora delbrückii*; *K.m.*, *Kluyveromyces marxianus*; *H.w.*, *Hansenula wingei*; *A.t.*, *Arxioma telluris*; *D.h.*, *Debaromyces hansenii*; *Z.b.*, *Zygosaccharomyces bisporus.* (**B**) Schematic representation of the structure of the *S.cerevisiae* ITS1 according to Yeh *et al.* (40). The stem–loop structure formed by the sequence shown in (A) is highlighted.

the plasmid to restore sectoring. Therefore, YOL080 clearly carries the mutation that is *sl* with *rrp5-Δ4* in these cells. Sequencing of the *sl* allele of YOL080 revealed a single mutation that changes the codon for Lys_{274} to a stop codon.

Recently, van Hoof *et al.* (19) showed that YOL080 encodes a member of a set of five related, non-essential, yeast proteins that includes Pan2p, the 3′→5′ exonuclease involved in initial shortening of the poly(A) tails of mRNA (36). The other four members were called Rex1p, Rex2p, Rex3p and Rex4p, the last one being the product of YOL080. The Rex proteins also contain the 3′→5′ exonuclease signature domain at their C-terminus (37,38). Rex1p–Rex3p were indeed found to be 3′→5′ exonucleases, which have distinct, but overlapping, roles in 3′-end maturation of several rRNA, snRNA and snoRNA species (19). A *rex4* null mutant, however, does not display any detectable disturbance in RNA maturation (19; our unpublished data), leaving the enzymatic activity of the Rex4p protein to be established.

We crossed strain YJV515 (*rrp5-Δ4*) with strain YAV41 in which *REX4* had been inactivated by insertion of the kanamycin gene (kindly provided by Dr van Hoof). Analysis of 20 different tetrads demonstrated that viable spores producing cells that were auxotrophic for histidine (thus containing the *rrp5-Δ4* allele) invariably were also sensitive to geneticine (indicating the presence of an intact *REX4* gene). This further supports the existence of a genetic interaction between the *RRP5* and *REX4* genes. A similar cross using strain YJV306 led to the same result, showing that absence of Rex4p is also *sl* with the *rrp5-Δ3* deletion (data not shown).

Inactivation of *REX4* restores normal ITS1 processing in the *rrp5-Δ* strains

To study the functional relationship between Rrp5p and Rex4p we used strain YJV154 in which the genomic, wild-type *RRP5* gene is under control of the inducible *GAL* promoter (31). This strain was crossed with the *rex4* null mutant YAV41 (19) to give FVY8C (*GAL-rrp5/rex4⁻*). The latter strain was then transformed with a plasmid carrying either the *rrp5-Δ3* or *rrp5-Δ4* mutant allele under control of the authentic *RRP5* promoter. Transformants were grown on galactose and then shifted to glucose-based medium to block production of wild-type Rrp5p. For the FVY8C transformants this causes growth to stop after ~23 h (data not shown). Total RNA was isolated immediately before and 24 h after the shift and analyzed by northern hybridization as well as primer extension analysis; the results are shown in Figures 5 and 6, respectively.

As is evident from Figure 5, in cells expressing wild-type Rrp5p the absence of Rex4p does not influence the formation of the mature rRNA species. When grown on galactose, the *REX4* (YJV154) and *rex4⁻* (FVY8C) cells have the same, normal ratio of 5.8_S:$5.8S_L$ rRNA as well as the same levels of 18S and 25S rRNA (Fig. 5A, C and D, compare lanes 5 and 7 with lanes 1 and 3, respectively). Also, northern hybridization did not detect any significant differences in the levels of the various processing intermediates between galactose-grown YJV154 and FVY8C transformants (data not shown), in agreement with previous observations (19). However, when the transformants are made dependent upon the Rrp5pΔ3 or Rrp5pΔ4 mutant protein by growing them on glucose, the lack of Rex4p does have a surprising effect: in the absence of the Rex4p protein the shift in the ratio of 5.8_S:$5.8S_L$ characteristic of the respective *rrp5-Δ* mutation does not occur (Fig. 5B, lanes 6 and 8). Moreover, the A4-E fragment, which can be clearly seen in the *rrp5-Δ3* and *rrp-Δ4* cells that carry an intact *REX4* gene (Fig. 5B, lanes 2 and 4), does not accumulate in their *rex4⁻* counterparts (Fig. 5B, lanes 6 and 8). Thus, inactivation of the *REX4* gene corrects the abnormal processing within ITS1 caused by the *rrp5-Δ3* and *rrp-Δ4* mutations. There is no change in the levels of the mature, high-molecular-weight rRNA species in the *rrp5-Δ3/rex4⁻* or *rrp5-Δ4/rex4⁻* mutants (Fig. 5C and D).

Figure 6 shows the results of primer extension experiments, which are in full agreement with the conclusion drawn from the northern hybridization data. Using primer *4* (Fig. 2A) we analyzed RNA isolated from YJV154 and FVY8C cells carrying the *rrp5-Δ3* or *rrp-Δ4* plasmid either before or 24 h after a shift from galactose to glucose. Cells containing the intact *REX4* gene show a clear reduction in the signal corresponding to the stop at A3 after the shift and the concomitant appearance of the stops corresponding to the A4 site (compare lanes 2 and 4 with lanes 1 and 3, respectively). However, in the *rex4⁻* transformants we do not see any change in the signals corresponding to the A3 and A4 cleavage sites upon repression of the wild-type *RRP5* gene (compare lanes 6 and 8 with lanes 5 and 7, respectively). We conclude that the Rex4p protein is essential for redirecting ITS1 processing in both the *rrp5-Δ3* and *rrp5-Δ4* mutant from the normal (A3) to the alternative (A4) pathway.

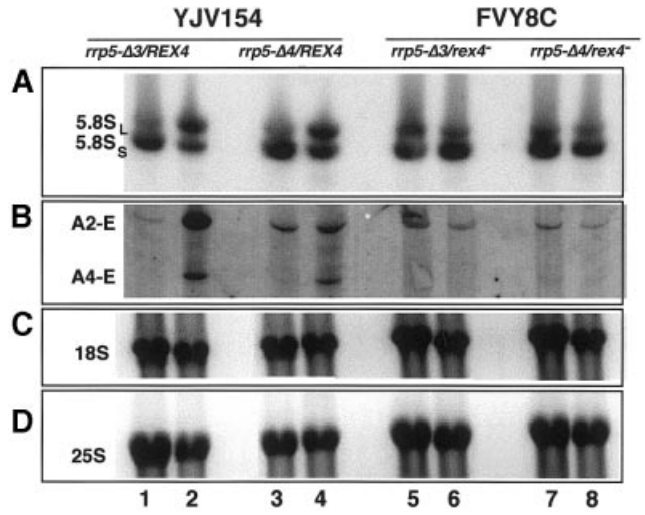


Figure 5. Effect of inactivation of *REX4* on formation of mature rRNA in *rrp5-Δ3* and *rrp-Δ4*. Strain FVY8C carrying a genomic wild-type *RRP5* gene under control of the *GAL* promoter, a *rex4* null allele and a plasmid-encoded *rrp5-Δ3* or *rrp-Δ4* gene under control of the authentic *RRP5* promoter was shifted from galactose- to glucose-based medium and total RNA was isolated immediately before and 24 h after the shift. The RNA was subjected to northern analysis using probes complementary to the mature 5.8S, 18S and 25S rRNAs [(**A**), (**C**) and (**D**), respectively] as well as probe *3* (see Fig. 2A) located directly upstream from cleavage site A3 (B). Strain YJV154, which is isogenic with FVY8C except for the presence of a wild-type *REX4* gene, was used as a control. Lanes 1, 3, 5 and 7, RNA from galactose-grown cells. Lanes 2, 4, 6 and 8, RNA from cells shifted to glucose for 24 h. The nature of the products detected is indicated.

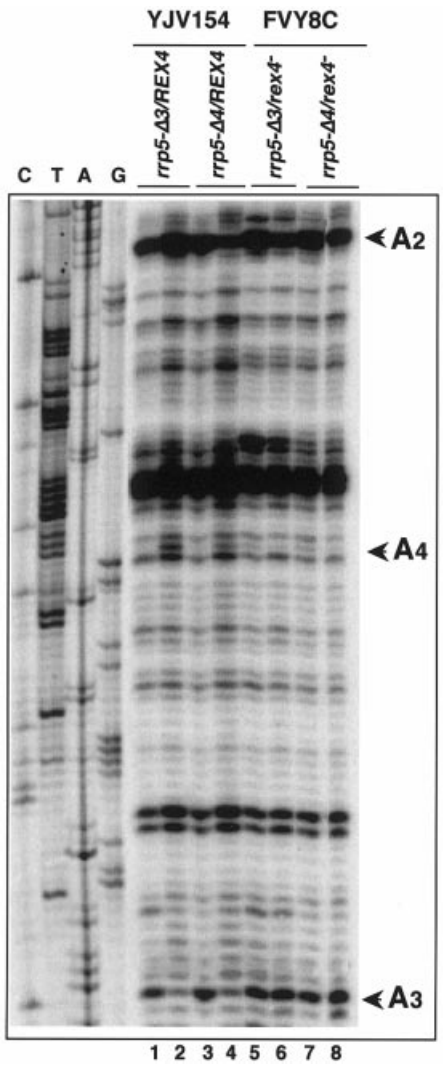


Figure 6. Effect of inactivation of *REX4* on ITS1 processing in *rrp5-Δ3* and *rrp-Δ4* mutant cells. Strain FVY8C carrying a genomic wild-type *RRP5* gene under control of the *GAL* promoter, a *rex4* null allele and a plasmid-encoded *rrp5-Δ3* or *rrp-Δ4* gene under control of the authentic *RRP5* promoter was shifted from galactose- to glucose-based medium and total RNA was isolated immediately before and 24 h after the shift. The RNA was subjected to primer extension analysis using probe *4*, located between sites A3 and B1 (see Fig. 2A). The position of the stops corresponding to cleavage sites A2, A3 and A4 is indicated. Lanes 1, 3, 5 and 7, RNA from galactose-grown cells. Lanes 2, 4, 6 and 8, RNA from cells shifted to glucose for 24 h.

Inactivation of *REX4* in cells dependent on the Rrp5pΔ3 or Rrp5pΔ4 protein leads to severe underproduction of 80S ribosomes

The observation that *rex4*⁻ cells expressing either of the Rrp5pΔ mutant proteins possess normal levels of the pre-rRNA intermediates and mature rRNA species came as a surprise in view of the *sl* phenotype of such cells. In order to see whether the *sl* phenotype might nevertheless be due to a disturbance in ribosome biogenesis we carried out sucrose gradient analysis of extracts from YJV154 (*GAL-rrp5/REX4)* as well as FVY8C (*GAL-rrp5/rex4*⁻) cells carrying the wild-type *RRP5* gene or either of the two *rrp5* deletion alleles on a plasmid. All strains were initially grown on galactose and then shifted to glucose-based medium for 24 h prior to preparation of the extracts. As shown in Figure 7 the *rrp5-Δ3* and *rrp-Δ4* mutations themselves already have an effect on ribosome biogenesis. The sucrose gradient profiles of glucose-grown YJV154 transformants that carry either of the plasmid-encoded *rrp5* deletion alleles reveal a significant deficit in 60S subunits apparent from the lower 60S and 80S peaks relative to the 40S subunit peak (Fig. 7, compare C and E with A). We have confirmed this by analyzing the polysome profile of these cells, which in the case of the *rrp5-Δ3* mutant clearly shows the presence of 'halfmer' polyribosomes, mRNA molecules carrying a single 40S subunit in addition to one or more 80S ribosomes, which is characteristic of the underproduction of 60S subunits (Fig. 7H). We did not detect halfmer polyribosomes in the *rrp5-Δ4* mutant (Fig. 7I) suggesting that the effect of this mutation on 60S subunit formation is less severe than that of its *Δ3* counterpart. This is in agreement with the relative growth rates of cells expressing either protein (31; this paper).

Introduction of the *rex4* null mutation into cells producing wild-type Rrp5p has no detectable effect on the ribosome profile (Fig. 7B). A distinct change can be seen, however, in glucose-grown FVY8C (*GAL-rrp5/rex4*⁻) cells expressing the Rrp5pΔ3 or Rrp5pΔ4 mutant protein (Fig. 7D and F). Apart from the decreased level of 60S subunits discussed above, these cells show a dramatic reduction in the amount of 80S ribosomes. Despite the absence of detectable abnormalities in pre-rRNA processing in these cells, therefore, ribosome biogenesis is severely disturbed, which explains the *sl*

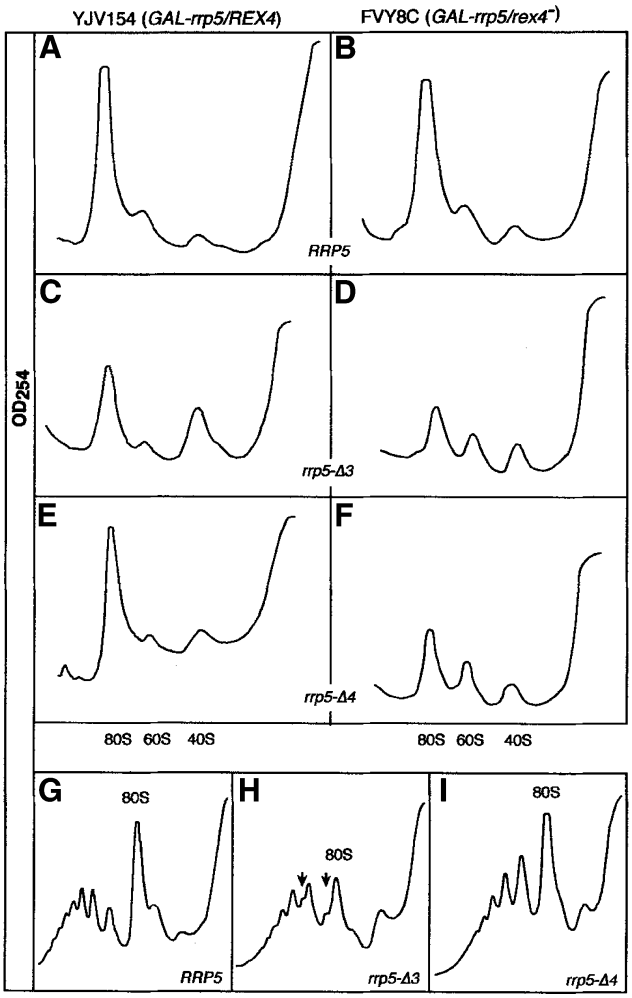


Figure 7. Effect of inactivation of the *REX4* gene on ribosome biogenesis in *rrp5-Δ3* and *rrp-Δ4* cells. Cultures of YJV154 (*GAL-rrp5/REX4*) and FVY8C (*GAL-rrp5/rex4⁻*) cells carrying either the wild-type *RRP5* gene or one of the *rrp5-Δ* deletion alleles on a plasmid were shifted from galactose- to glucose-based medium and extracts were prepared 24 h later. 20 OD_{260} units of extract were analyzed on 15–35% (**A**–**F**) or 10–50% sucrose gradients (**G**–**I**). Sedimentation is from right to left. (A, C and E) Extracts from strain YJV154 carrying the wild-type *RRP5* (A), *rrp5-Δ3* (B) or *rrp-Δ4* (C) allele. (B, D and F) Extracts from strain FVY8C carrying the wild-type *RRP5* (B), *rrp5-Δ3* (D) or *rrp5-Δ4* (F) allele. (G–I) Polysome profiles for YJV154 carrying the wild-type *RRP5* (G), *rrp5-Δ3* (H) or *rrp5-Δ4* (I) allele. The arrows in panel H indicate the 'halfmers'.

phenotype of combining the *rrp5-Δ3* or *rrp5-Δ4* and *rex4⁻* mutations.

DISCUSSION

Genetic depletion of Rrp5p blocks synthesis of both 18S rRNA, requiring the snoRNP-dependent cleavages at A0–A2, as well as $5.8S_S$ rRNA, which depends upon the RNaseMRP-directed cleavage at A3. This led to the hypothesis that the major role of Rrp5p is to ensure functional integration of the many *trans*-acting factors required for accurate and efficient pre-rRNA processing at these sites (30). Subsequent mutational analysis (31,32) demonstrated that the C-terminal region of the protein, containing seven TPR motifs, is specifically involved in 18S rRNA synthesis, whereas the N-terminal region, encompassing 12 S1 RNA-binding motifs, is crucial for production of the $5.8S_S$ rRNA. Thus, the C-terminal domain might interact with the snoRNP complex that carries out the early processing cleavages, while the N-terminal domain assists RNase MRP. The data presented in this paper constitute direct experimental proof for the latter suggestion as they demonstrate that removal of either S1 motifs 3–5 (*rrp5-Δ3)* or 5–8 (*rrp5-Δ4*) leads to a complete block in the RNase MRP-directed cleavage at site A3 (Fig. 3).

Interestingly, our experiments reveal a second effect of the *rrp5-Δ3* and *rrp-Δ4* mutations, namely the occurrence of a hitherto unobserved processing event in ITS1 at a site about midway between A2 and A3 which we have designated A4 (Fig. 3). The simultaneous presence of substantial amounts of an A4-E fragment (Fig. 2D, E and F), normal amounts of the D-A2 fragment (Fig. 2H) and, in particular, the detection of the A4 site in primer extension experiments using probes *6* and *7* complementary to different regions of ITS2 clearly support the conclusion that the *rrp5-Δ3* and *rrp-Δ4* mutations cause the processing machinery to bypass A3 and instead to convert the $27SA_2$ pre-rRNA into an alternative, novel $27SA_4$ precursor species.

The currently available data do not allow us to decide whether formation of the $27SA_4$ species is an endo- or an exonucleolytic event. However, we favor the former possibility for the following reasons. First, the processing intermediate having its 5′-end at A4 accumulates in the presence of LiCl, which strongly inhibits the major 5′→3′ exonucleases Xrn1p and Rat1p known to be involved in pre-rRNA processing (Figs 2 and 3). Second, the $27SA_2$ pre-rRNA, the probable immediate precursor of the $27SA_4$ species, appears to be a poor substrate for these exonucleases, in any case, at least under normal conditions (13,14). Finally, as shown in Figure 4, the A4 site is located in a region of ITS1 that has been relatively well conserved with respect to both primary and secondary structure over a wide spectrum of yeast species, suggesting that it could be the recognition site for an endonuclease, the nature of which remains to be identified. There is no obvious structural similarity of this region with those containing other known processing sites within the pre-rRNA. It should also be noted that processing at A4 might be less precise than the standard processing events. In all cases we observe a set of three bands corresponding to consecutive nucleotides, whose relative intensity varies somewhat from one experiment to another (Figs 3 and 6).

Although the *rrp5-Δ3* and *rrp-Δ4* deletions cause identical changes in ITS1 processing the fate of the resulting precursors differs in the two mutants. In the *rrp5-Δ3* strain subsequent processing almost exclusively follows the 'long' pathway leading to $5.8S_L$ rRNA. In the *rrp5-Δ4* mutant, on the other hand, substantial processing still occurs via the 'short' pathway, presumably by exonucleolytic degradation of the $27SA_4$ precursor, resulting in ~40% $5.8S_S$ rRNA. We propose that the presence of mutant Rrp5p protein causes a structural alteration in the processing complex that reduces either the rate of entry of the exonucleases or the rate of exonucleolytic digestion, allowing processing at site $B1_L$ to get the upper hand. Clearly, the extent of this negative effect on $B1_S$ processing is different for the two mutant proteins, being largest for the *rrp5-Δ3* mutation.

A synthetic lethality screen using the *rrp5-Δ4* mutation resulted in the isolation of the *REX4* gene, which encodes a protein belonging to a family of non-essential 3′→5′ exonucleases. Although other members of this family were shown to be involved in pre-rRNA processing, no evidence for such a role was found in the case of the Rex4p protein (19). The data presented in Figures 5 and 6, however, clearly demonstrate that inactivation of the *REX4* gene does have a rather surprising effect on pre-rRNA processing in strains expressing either of the mutant Rrp5pΔ proteins. Both the *rrp5-Δ3/rex4⁻* and *rrp5-Δ4/rex4⁻* double mutants show normal ITS1 processing as well as a wild-type $5.8S_S$:$5.8S_L$ ratio. We conclude that the change in ITS1 processing induced by the *rrp5-Δ3* and *rrp-Δ4* mutations requires intact Rex4p and that in the absence of Rex4p the mutant processing complex containing either mutant Rrp5p protein regains the ability to direct RNaseMRP to the A3 site. The molecular basis for this phenomenon remains a matter of speculation. Rex4p could participate directly in ribosome biogenesis, a conceivable hypothesis in view of the fact that its human homolog resides in the nucleolus (39). Another possibility is that Rex4p plays a role in the formation of an as yet unidentified *trans*-acting factor that forms part of the processing complex containing Rrp5p. In wild-type cells, however, the role of Rex4p is not critical.

The lack of any detectable abnormalities in pre-rRNA processing in the *rrp5-Δ3/rex4⁻* and *rrp5-Δ4/rex4⁻* double mutants left us without an obvious explanation for the *sl* phenotype of these mutants. Therefore, we considered the possibility of a defect in ribosome assembly, which for obvious reasons we studied in strains in which the wild-type *RRP5* gene is conditionally expressed. Sucrose gradient analysis demonstrated that in fact the *rrp5-Δ3* and *rrp-Δ4* mutations by themselves already cause a significant defect in 60S subunit assembly. Extracts from cells expressing the Rrp5pΔ3 protein show a clear deficit in the large, relative to the small, subunits as well as the characteristic presence of halfmer polyribosomes (Fig. 7). This could be a consequence of the almost exclusive presence of $5.8S_L$ rRNA in these cells which might be assembled less efficiently than its smaller counterpart, similar to 3′-extended 5.8S rRNA (17). This idea is supported by the fact that the deficit in 60S subunits, in particular as judged from the absence of detectable amounts of halfmers, is less severe in the *rrp5-Δ4* mutant, which contains a higher proportion of $5.8S_S$ rRNA (31; this paper). On the other hand, the structural abnormality of the Rrp5p protein might also directly affect 60S subunit assembly in the mutant cells.

Sucrose gradient analysis of extracts prepared from the *rrp5-Δ3/rex4⁻* and *rrp5-Δ3/rex4⁻* double mutants revealed a very low amount of 80S ribosomes (Fig. 7). The combination of mutant Rrp5p and the absence of Rex4p, therefore, appears to cause a severe defect in ribosome assembly, which would explain the *sl* phenotype of the double mutants. This finding further supports the hypothesis that Rex4p is involved in ribosome biogenesis, although its role only becomes manifest in the presence of the mutant Rrp5p proteins. We are presently studying the role of Rex4p in ribosome biogenesis further using both genetic and biochemical approaches.

In summary, the data presented in this paper firmly establish the role of the S1 domain of Rrp5p in assisting RNaseMRP in its cleavage at site A3. The involvement of Rrp5p in this processing step seems to be rather complex, however, because the requirement for the S1 motifs in question can be abrogated by removing Rex4p. The data also indicate a further role for Rrp5p, either directly or indirectly, in the subsequent exonucleolytic processing to site $B1_S$ and demonstrate a negative effect of the two deletion mutations on 60S subunit biogenesis. Furthermore, both genetic and biochemical evidence clearly indicates the involvement of Rex4p in the assembly of yeast ribosomes, albeit in an unusual manner.

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